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Full Length Research Paper

Production and purification of polyclonal antibodies against 34 kDa protein (P34) of *Mycobacterium avium* subsp. *Paratuberculosis*

Hafezeh Alizadeh[1], Rasool Madani[2*], Narges Kavid[2], Fariba Golchinfar[2] and Tara Emami[2]

[1]Department of Biology, Science & Research Branch, Islamic Azad University, Tehran-Iran.
[2]Department of Biochemistry and Proteomics, Razi Vaccine and Serum Research Institute, Karaj-Iran.

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Paratuberculosis caused by *Mycobacterium avium* subsp. *paratuberculosis* (MAP), is a chronic enteritis in ruminants. Among molecular components of MAP, protein P34 was identified as specific and immunodominant. Here, we describe the production of polyclonal antibodies with defined specificity for P34. Polyclonal antibodies were generated from New Zealand white rabbit. Animals were immunized at a certain time period with purified P34, MAP antigens and Freund's adjuvant. Antibodies were purified from serum by ion exchange chromatography. Western blotting analysis was used for evaluation of interaction between 34 kDa protein and antibodies.

Key words: 34 kDa antigen, Paratuberculosis, Polyclonal antibodies.

INTRODUCTION

Mycobacterium avium subsp. paratuberculosis (MAP) is the agent of paratuberculosis or Johne's disease (JD), a granulomatous enteritis in ruminants, that causes economic losses to the dairy industry (Berger et al., 2007; Santema et al., 2011). Despite its heavy economic burden, there are still no efficacious vaccination programs against Johne's disease (Bannantine et al., 2008) characterized by intermittent diarrhea, weight loss and eventual death Mundo et al., 2008). Diagnostic methods for paratuberculosis rely on the ability of the test to detect infected animals (Mutharia et al., 1997). Ruminants affected by JD develop strong immunological reactivity against *M. avium* subsp. *paratuberculosis* antigens (Berger et al., 2006). Current immuno-diagnostics tests are based on crude antigen mixtures with poor sensitivity due to high similarity between MAP and other *mycobacterial* antigens (Kavid et al., 2012). Some MAP antigens are now being evaluated for the development of new more sensitive diagnostics tests for paratuberculosis (Willemsen et al., 2005) and several studies with these antigens have been performed to set up diagnostic tests of paratuberculosis in livestock (Gioffre et al., 2006). Therefore, it is of interest to identify and characterize specific antigens of MAP and evaluate the role of these molecules as modifiers of cellular and humoral immunity (Mutharia et al., 1997). Antigens with molecular weight of 28 to 45 kDa are the most antigenic for both cellular and humoral immunity (De Kesel et al.,

*Corresponding author. E-mail: mr_madani@yahoo.com. Tel: 0098-21-34570038. Fax: 0098-26-34552194.

1993). De Kesel et al. (1992) and Gilot et al. (1993) identified a 34-kDa protein from the A36 complex, the major antigenic complex of *M. avium* subsp. *paratuberculosis* (Coetsier et al., 1998). P34 was specific and shown to be immunodominant (Malamo, 2011). The carboxy terminus of this protein contain species-specific epitopes (Malamo, 2006). The identification of epitopes on MAP P34 is important to determine the role of bovine antibodies in the response against MAP infection (Ostrowski et al., 2003) so P34 kDa protein has become a notified subject for more studies. The aim of this study was to characterize the humoral immune response induced by immunization with MAP antigens in New Zealand white rabbit and evaluate the immunogenicity of P34.

MATERIALS AND METHODS

The purified 34 kDa protein from *M. avium paratuberculosis* (ATCC19698) was obtained from the Department of Biochemistry and proteomics, Razi Vaccine and Serum Research Institute Karaj, Iran [7]. DEAE-Cellulose columns were purchased from Pharmacia (Sweden). Protein markers were obtained from BioRad (Hercules, USA). Other reagents and chemicals were of analytical grade from Fluka and Merck.

Animal

New Zealand white female rabbit was supplied from the Laboratory Animal Breeding Unit of Razi Vaccine and Serum Research Institute, Iran.

Protein determination

Protein concentration of purified P34 kDa was measured by the method of Lowry et al., using BSA as standard (Lowry et al., 1951).

Immunization of rabbit with whole-cell extract of MAP

A New Zealand white rabbit was immunized four times at 14 days intervals with whole-cell extract and purified p34 to obtain polyclonal antibodies. So, for per injection, 600 µl of MAP antigens (300 µl whole cell extract were mixed with 300 µl purified p34) were emulsified with equal volume of Freund's adjuvant (Sigma). For first injection, antigens were used in Freund's complete adjuvant and emulsion was administered subcutaneously. The second, third and fourth injection were performed on days 14, 28 and 42 in Freund's incomplete adjuvant (Sigma). Blood sample was taken 2 weeks later from rabbit and serum was prepared (Shin et al., 2009).

Ig preparation by ammonium Sulfate

The prepared serum (3 ml) was precipitated by ammonium sulfate (35%). The suspension were centrifuged at 10000 × *g* for 30 min at 4°C, the supernatant was removed and the precipitate suspended in the PBS buffer 0.01 M (1.5 µl) and sample dialyzed against PBS buffer (pH 6.5) overnight on a magnetic stirrer at 4°C (Mutharia et al., 1997)

Purification of IgG

Ion-exchange (DEAE-cellulose) chromatography was used for purification of IgG. Ig passed through the column (1.5 × 16 cm) and washed in two steps using PBS buffer 0.01 M for first time and PBS 0.1 M buffer at pH 6.5 for the second washing step. The column was run, by upward flow, at 20 ml/h rate and fractions of 1 ml were collected. The effluent was monitored at 280 nm (Pharmacia Biotech 2000). Fractions concentrations containing IgG were determined with Lowry assay (Lowry et al., 1951).

SDS-PAGE analysis of IgG

The purity of rabbit IgG preparation was checked using Bio-Rad 10% sodium dodecyl sulphate gel electrophoresis (SDS-PAGE) under reduced conditions as described by Laemmli (1970). 50 µl IgG (9 mg/ml) was solubilized in 50 µl of sample buffer (0.0625 M Tris-HCL, pH 6.8, 10% glycerol, 2% SDS, 0.006% bromophenol blue, 5% β-mercaptoethanol), and was boiled for 10 min and loaded on the electrophoresis gel. Electrophoresis was carried out at a constant current of 110 V for 1 h. After separation, the proteins bands were stained by Coomassie brilliant blue staining solution (0.1% Coomassie blue in 70:15:15 methanol: water: acetic acid), and destained in destaining buffer (70:15:15 methanol: water: acetic acid).

Double-Immunodiffusion Assay

The double-immunodiffusion was performed in 1.2 % agar gel prepared in the PBS 0.01 M buffer at pH 8.6 with polyclonal antisera against MAP antigens used in this work. MAP antigens were poured into central wells, and serially diluted antisera were put into peripheral ones. The Coomassie Brilliant Blue R-250 solution (0.1% Coomassie blue in 70:15:15 methanol: water: acetic acid) was used for the gel fixation and staining. The gel was destained by acetic acid solution (70:15:15 methanol: water: acetic acid) for 20 min at room temperature with shaking (Nikolayenko et al., 2005).

SDS-PAGE and Western blot analysis

50 µl of the MAP antigens (34.65 mg/ml) were mixed in 50 µl of sample buffer (0.0625 M Tris-HCL, pH 6.8, 10% glycerol, 2% SDS, 0.006% bromophenol blue, 5% β-mercaptoethanol), and was boiled for 5-10 min. Sample was separated in Bio-Rad 10% polyacrylamide gel electrophoresis at 100 V for 1.5 h by Laemmli's method (Laemmli, 1970). For immune blotting, protein bands were transferred to nitrocellulose membrane in a semidry unit (Bio-Rad PAC 1000) at 10 V for 30 min. The membrane was blocked with 3% BSA in PBS 0.01 M buffer (blocking buffer) for 2 h at 4 °C. After washing 3 times with PBS-T (500 cc PBS 0.01 M, 250 µl Tween 20), membrane was incubated in rabbit purified anti-MAP IgG (1:20 dilution) for 1.5 h with shaking at room temperature and then incubated overnight at 4°C. After washing 4 times with PBS-T, the membrane was incubated with anti-rabbit HRP IgG conjugate (sigma, 1:1000 dilution in PBST) for 1 h at room temperature and again washed for 3 times with PBS-T. Finally, the chloronaphtol peroxidase [methanol (16 ml), chloronaphtol (18 ml), PBS (24 ml), peroxidase (6 µl)] was added to the membrane and incubated for 15 min to identify the developed band of polyclonal anti-MAP IgG (Yabe et al., 1995).

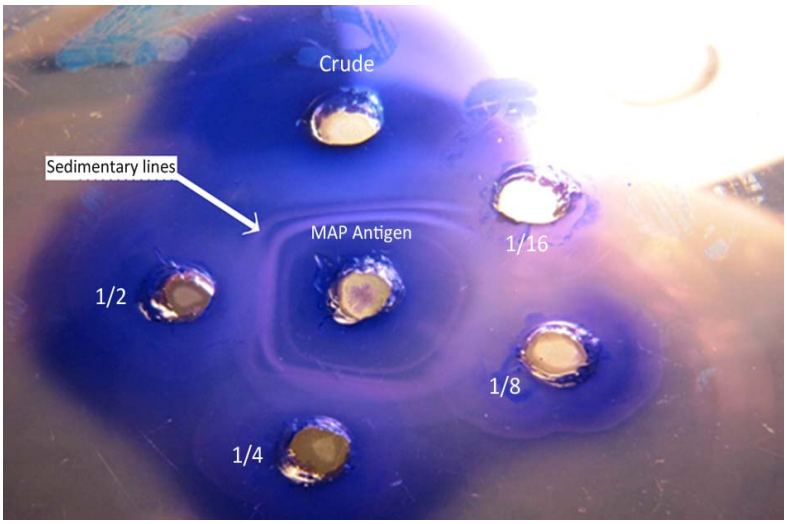

Figure. 1. Double immuno-diffusion test were applied with anti-MAP IgG in crude, 1:2, 1:4, 1:8 and 1:16 dilutions and precipitation lines between antibodies and antigens.

RESULTS

Estimation of P34 concentration

The concentration of P34 kDa was 0.096 mg/ml.

Polyclonal antibodies titer

Usually antibodies titers increase every new immunization. In this experiment the best antibodies titers were obtained from the rabbit serum collected after the fourth injection. The interaction of polyclonal anti-MAP IgG was evaluated by double-immunodiffusion test with crude, 1:2, 1:4, 1:8 and 1:16 dilutions of IgG and 3 sharp lines of precipitation were shown among MAP antigens and different titer of antibodies (till to 1:16 dilution) (Figure 1).

Purification of anti-MAP IgG

Purification of rabbit anti-MAP IgG was carried out by ion exchange chromatography on DEAE-Cellulose. In this step, as shown in the chromatogram (Figure 2) of rabbit immune-globulins, only one peak was gained. The fractions containing IgG were collected and the whole protein (IgG) concentration was obtained 9 mg/ml by Lowry assay.

SDS-PAGE analysis of purified IgG

For separating rabbit IgG, SDS-PAGE was performed under reducing condition and IgG band was evaluated in Coomassie blue stain. Figure 3 shows a single band with molecular weight about 50 kDa corresponding to rabbit IgG heavy chain (Figure 3).

Western blot analysis of MAP antigens

Western blot analyses of MAP antigens were performed with polyclonal antibodies of rabbit which showed several bands. These bands showed positives reactions between rabbit antibodies and MAP with the 34 kDa molecular weight protein identified as a sharp band (Figure 4). The specific band for P34 showed the immunodominant specificity of this protein against rabbit anti-MAP IgG.

DISCUSSION

Several antigenic proteins of MAP have been described in the pathogen and have been performed to set up diagnostic tests. Amongst whole antigens of MAP, P34 has been cloned and shown to be immunodominant antigen and have been fully characterized previously which is a good candidate to use in immunological analysis (Kavid et al., 2012; Coetsier et al., 1998). P34 has a significant role in formation of granulomata and other hypersensitivity type responses manifested in JD (Kavid et al., 2012). The carboxyl terminus of P34 containing specific epitopes with respect to MAP has been used as antigen in an ELISA for the specific diagnosis of bovine paratuberculosis (Coetsier et al., 1998). Coestier and colleagues described the preparation of polyclonal and monoclonal antibodies, directed against the 13.6 kDa carboxyl-terminal portion of the rcP34 of MAP (Malamo, 2011). Malamo (2011) used carboxyl terminous from the rcP34 of *M. paratuberculosis* to produce

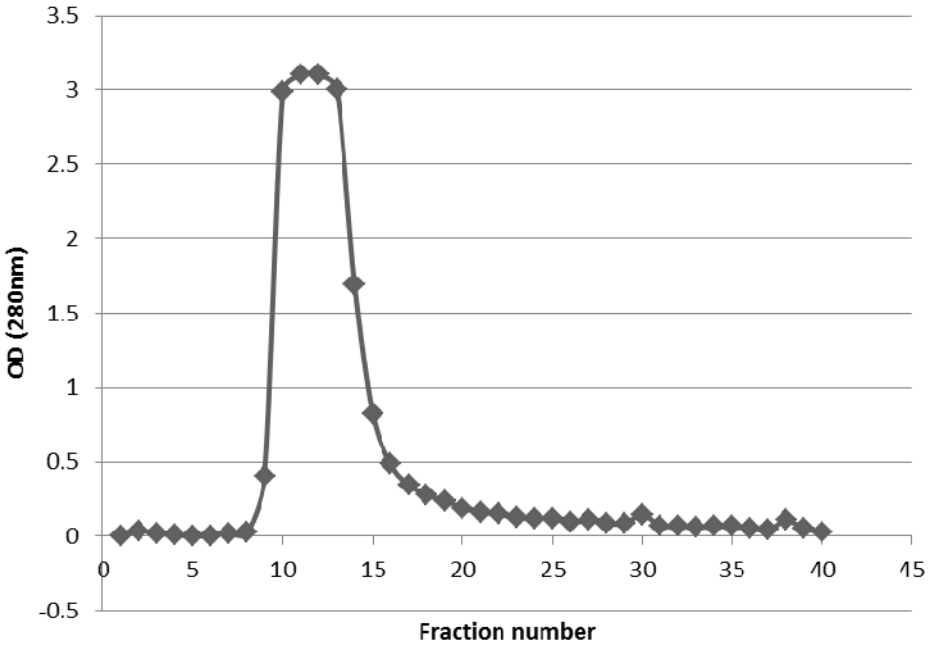

Figure. 2. Immunoglobulin solution was applied on DEAE-Cellulose column (1.5 × 16 cm). One peak was obtained from fraction 11 to fraction 17.

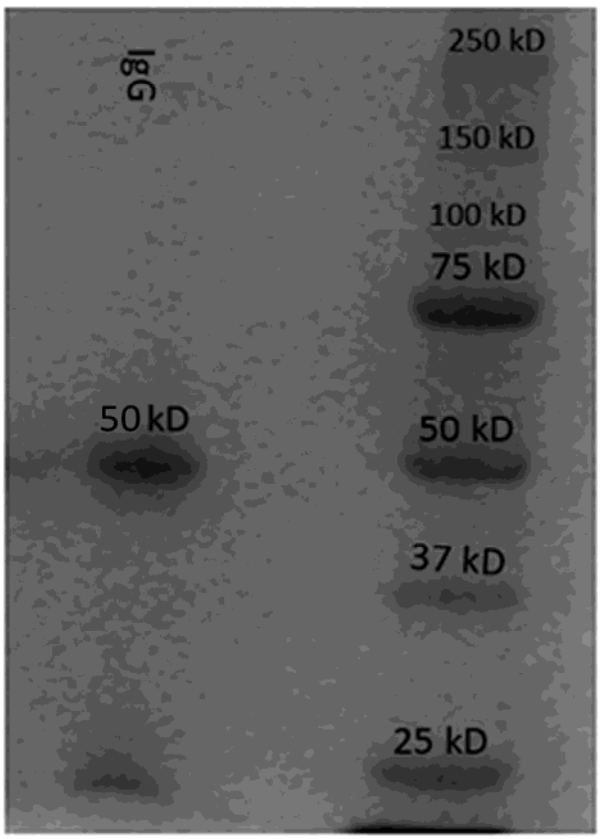

Figure. 3. SDS-PAGE: Pure IgG is shown as a single band with molecular weight about 50 kDa corresponding to rabbit IgG heavy chain.

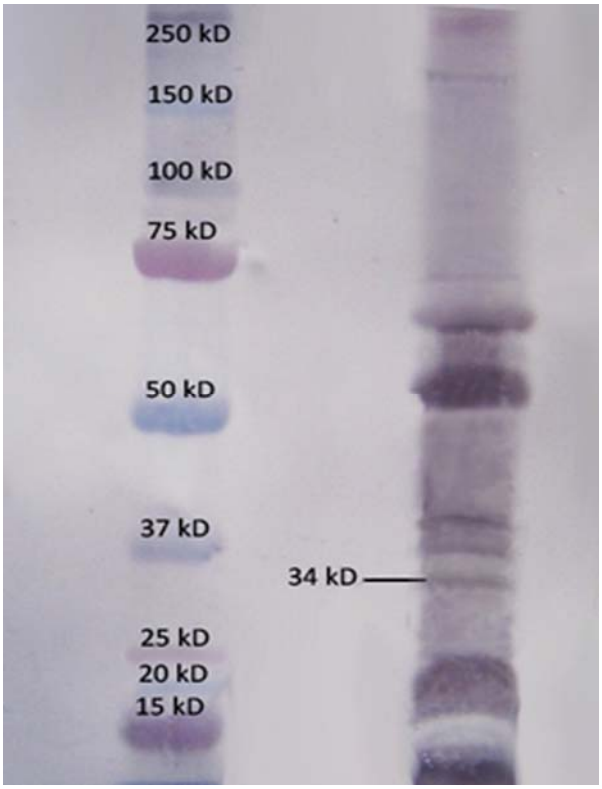

Figure 4. Western blot analysis of *Mycobacterium avium paratuberculosis* by rabbit anti-MAP IgG showed positive reactions between antigens and antibodies that P34 identified as a sharp band and this band showed the immunodominant specificity of P34.

monoclonal antibodies against MAP (Malamo, 2011). Purification of native P34 kDa protein from cell wall extracts is difficult because of aggregation and interaction of this protein with MAP cell wall proteins. P34 was previously purified by electroelution method in Biochemistry and proteomics Department of Razi Institute (Kavid et al., 2012) and in this study we used this purified protein and whole cell extracts of MAP containing P34 for produced polyclonal antibodies in rabbit. Antibodies are an important tool for investigation of protein expression and function. Polyclonal antibodies enhanced analytical sensitivity because they are directed against multiple surface antigens. Immunization of rabbit with MAP antigens was performed and blood was collected. We used ion-exchange chromatography for the purification of rabbit IgG. With Western blotting, we demonstrate the specificity of polyclonal antibodies against P34 of *M. avium paratuberculosis* and identified the immunogenicity of p34. A previous study has reported the potential of polyclonal rabbit anti-MAP antibodies to be used as a separation tool to detect MAP in milk or fecal samples (Khare et al., 2004). So we purified rabbit IgG polyclonal antibodies by ion-exchange chromatography to evaluate the reactivity of P34 by Double-immunodiffusion and western blot analysis. Double-immunodiffusion of whole MAP proteins showed three precipitate lines; these lines indicate high reactivity of antigens with rabbit anti-MAP IgG. In addition, we performed western blot analysis and result has showed several bands to MAP antigens and p34 kDa appeared as a sharp band. Moreover, the band of P34 suggests a significant role of P34 in the immunobiology of infection. Western blot confirm that P34 was immunodominant with rabbit anti-MAP IgG. In conclusion, the identification of polyclonal antibodies employing western blot analysis is of importance in studying the MAP disorder. The polyclonal anti-MAP IgG produced in this work were specific for P34 of MAP and these polyclonal antibodies could be useful as a specific antibody for future study.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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